Evidence for Specific Autoimmunity Against Sympathetic and Parasympathetic Nervous Tissues in Type 1 Diabetes Mellitus and the Relation to Cardiac Autonomic Dysfunction

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There is growing evidence for the involvement of immunological factors in the pathogenesis of cardiac autonomic dysfunction in Type 1 diabetes mellitus (DM). To evaluate the presence of autoantibodies against autonomic nervous tissues and their relationship with tests of autonomic function, 64 newly diagnosed and 142 long duration Type 1 DM patients were investigated for sympathetic and parasympathetic ganglia (CF-SG and CF-PSG) autoantibodies with a complement-fixing indirect immunofluorescence technique. Five cardiac reflex tests were performed to assess autonomic function. Fifty-seven patients with neurological diseases other than diabetic neuropathy and 131 healthy control subjects were also tested for CF-SG and CF-PSG autoantibodies. CF-SG autoantibodies were observed in 47 (23 %) and CF-PSG autoantibodies in 21 (10 %) of 206 Type 1 DM patients ($p < 0.001$). In contrast, these autoantibodies were detected in 3 (5 %) and 1 (2 %) of patients with non-diabetic neurological diseases and 3 (2 %) and 4 (3 %) of control subjects ($p < 0.01$, $p < 0.05$, $p < 0.0001$, $p < 0.05$ vs Type 1 DM patients). All except two Type 1 DM patients with CF-PSG autoantibodies also presented with CF-SG autoantibodies. In diabetic patients with long duration, CF-SG autoantibodies were more frequent in patients with ECG-based cardiac autonomic neuropathy (CAN; $\geq$2 of 5 cardiac reflex tests abnormal) compared to patients without CAN although this did not reach statistical significance (29 % vs 17 %, $p = 0.06$). However, 4 (80 %) of 5 newly diagnosed and 23 (32 %) of 73 established Type 1 DM patients with abnormalities in heart rate variation during deep breathing and/or standing from lying presented with CF-SG autoantibodies compared to 12 (25 %) of 58 newly diagnosed ($p < 0.05$) and 7 (11 %) of 63 established Type 1 DM patients ($p < 0.01$), in whom both tests were normal. The results suggest that autoimmune factors contribute to the pathogenesis of cardiac autonomic dysfunction in Type 1 DM and that autoantibodies against sympathetic and parasympathetic nervous tissues are relatively specific for Type 1 DM. © **1998 John Wiley & Sons, Ltd.**

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KEY WORDS **Type 1 diabetes mellitus; autonomic neuropathy; anti-sympathetic ganglia autoantibodies; anti-parasympathetic ganglia autoantibodies; cardiac reflex tests**

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Introduction

It is suggested that autoimmune factors are involved in the pathogenesis of cardiac autonomic dysfunction of Type 1 diabetes mellitus (DM).[1–3] Autoantibodies against sympathetic ganglia cells (CF-SG autoantibodies) have been detected in established, newly diagnosed, and prediabetic Type 1 DM patients.[4–6] Using 123-I-MIBG scintigraphy to assess cardiac sympathetic denervation in established Type 1 DM patients, we observed an association between CF-SG autoantibodies and 123-I-MIBG uptake.[7] Recently, we demonstrated that CF-SG autoantibodies tended to occur more frequently in long

Abbreviations: CAN cardiac autonomic neuropathy, CF-SG complement fixing sympathetic ganglia, CF-PSG complement fixing parasympathetic ganglia, HRV heart rate variation

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duration Type 1 DM patients with a diagnosis of ECG-based cardiac autonomic neuropathy (CAN, 34 %) compared to those without (18 %).[8] We also showed that CF-SG autoantibodies can be detected independently of autoantibodies to the diabetic-specific antigens glutamic acid decarboxylase (GAD) and tyrosin phosphatase (IA-2/ICA 512) and that the occurrence of CF-SG autoantibodies is independent from the presence of islet cell autoantibodies.[8]

In order to investigate further a possible pathogenic association between autoantibodies against autonomic ganglia cells and diabetic autonomic neuropathy, we have extended the studies on CF-SG autoantibodies in Type 1 DM patients and applied the complement-fixing indirect immunofluorescence technique to detect autoantibodies against parasympathetic ganglia (CF-PSG). We directly compared the titres of CF-SG autoantibodies with the individual parameters of cardiac reflex tests. The specificity of these autoantibodies was explored not only testing healthy control subjects but also patients with neurological diseases other than diabetic neuropathy.

Patients and methods

Patients

Four study groups were investigated for CF-SG and CF-PSG autoantibodies:

1. 64 patients with newly diagnosed Type 1 DM;
2. 142 long duration, established Type 1 DM patients;
3. 57 patients with neurological diseases other than diabetic neuropathy;
4. 131 healthy control subjects.

The clinical characteristics of the study groups are shown in Table 1. Diagnosis of Type 1 DM was based on WHO criteria.[9] All patients were on intensified insulin therapy which included four or more daily subcutaneous insulin injections or insulin administration by an external pump. In none of the patients with non-diabetic neurological diseases and in none of the control subjects was there any family history of Type 1 DM. Sera of the patients with non-diabetic neurological diseases were collected at the Department of Neurology (University of Erlangen, Germany). The diagnosis of these patients is specified in Table 2. Diagnosis of neuropathy in the various disorders was based on nerve conduction velocities and electromyograms. Nerve conduction velocities were determined from the right sided median (motor and sensory), peroneal (motor and sensory), and sural (sensory) nerve. Age-related normal ranges were used.[10] Diabetes mellitus was excluded by an oral glucose tolerance test[11] and determination of the HbA_{1c} (non-diabetic range 4.4–6.4 %).

Patients with a history of coronary heart disease, myocardial infarction or arrhythmias were excluded. Patients and control subjects were taking no medication known to interfere with cardiac function.[12]

Table 1. Clinical characteristics of the four study groups

	Newly diagnosed Type 1 DM patients ($n = 64$)	Long duration Type 1 DM patients ($n = 142$)	Patients with neurological diseases other than diabetic neuropathy ($n = 57$)	Control subjects ($n = 131$)
Male/female (n)	39/25	78/64	40/17	62/69
Age (yr)	28 ± 7	37 ± 13[a]	59 ± 14[b]	29 ± 6[c]
Body mass index (kg m^{-2})	21.3 ± 3.1	23.4 ± 2.5	25.9 ± 4.2	21.6 ± 1.9
Duration of diabetes (yr)	–	20 ± 11	–	–
HbA_{1c} (%)	11.7 ± 2.3	8.9 ± 2.0	5.4 ± 0.5	–
Background/ proliferation retinopathy (n)	0	68/11	–	–
Micro-/macro-albuminuria (n)	5/0	22/20	–	–

[a] $p < 0.001$ vs newly diagnosed Type 1 DM patients; [b] $p < 0.001$ vs newly diagnosed and long duration Type 1 DM patients; [c] $p < 0.001$ vs long duration Type 1 DM and patients with neurological diseases other than diabetic neuropathy.

Table 2. Patients with neurological diseases other than diabetic neuropathy ($n = 57$)

n	Diagnosis	positive for CF-SG (n)	CF-PSG (n)
13	Amyotrophic lateral sclerosis	1	0
2	Multifocal motor neuropathy	0	0
2	Guillan-Barre syndrome	0	0
2	Mononeuritis multiplex	0	0
1	Alcoholic neuropathy	0	0
1	Vasculitis	0	0
1	Parainfectious neuropathy	0	0
1	Thyroiditis	1	1
1	Hereditary neuropathy	0	0
1	Spinal muscular atrophy	0	0
32	Neuropathy of unknown aetiology	1	0

Assay for Sympathetic and Parasympathetic Ganglia Autoantibodies

An indirect complement-fixing immunofluorescence technique as previously described for detection of CF-SG autoantibodies[8] was applied to test sera for CF-SG and CF-PSG autoantibodies. Cryostat sections (5 μm) of snap frozen rabbit superior cervical sympathetic and parasympathetic ganglia were used as substrates. Identification of the different ganglia was performed with haematoxylin–eosin stain. Four dilutions of a positive reference serum, a negative serum, and PBS (control for endpoint-dilution) were tested in each assay as controls. All sera were tested in a blinded fashion, and intensity of fluorescence was scored by two independent observers

on a scale from 0 to 3. A score of 1 was used as the cut-off for positivity of CF-SG and CF-PSG autoantibodies (95th percentile of 131 control subjects).

For quantitative evaluation of CF-SG autoantibodies, patients' sera were diluted in PBS in comparison to the positive reference serum arbitrarily defined as 80 units. Figure 1 demonstrates the standard curve for the positive reference theorem. The upper limit of normal range was an antibody titre of 6 units, as defined by the 97th percentile of endpoint titres of the control subjects ($n = 131$).

Assessment of Cardiac Autonomic Function

As previously published,[13] five cardiac reflex tests were performed in diabetic patients to assess cardiac autonomic function: Heart rate variation at rest and during deep breathing, with calculation of the coefficients of variation; immediate heart rate response to standing expressed by the max/min 30:15 ratio; Valsalva manoeuvre with calculation of the ratio of the longest RR-interval after and the shortest RR-interval during the manoeuvre (Valsalva ratio); and the difference between lying and standing systolic blood pressure. The evaluation of the individual parameters was performed according to age-related normal ranges.[14] Criterion for an abnormal blood pressure response was a systolic blood presssure drop on standing of $\geq$30 mmHg.[15] ECG-based cardiac autonomic neuropathy (CAN) was defined as two or more abnormal cardiac reflex tests.

Statistical Analysis

Data are expressed as mean value $\pm$ SD. Differences in prevalence were compared using χ^2-test or Fisher's exact test. The parameter-free Spearman's rank test was used for correlation coefficients. Differences between groups were tested with the parameter-free Mann–Whitney test. A p-value of <0.05 was considered significant.

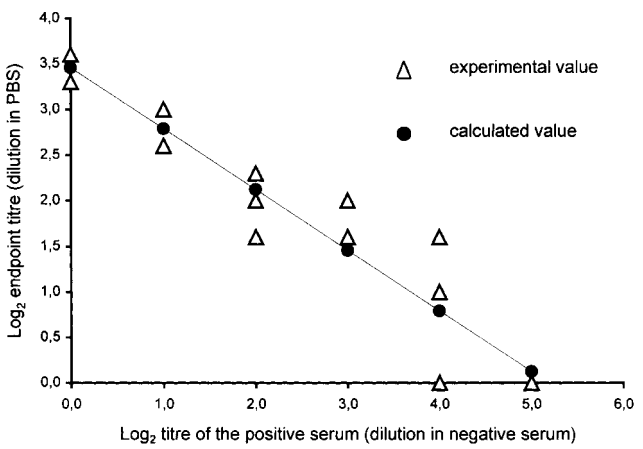

Figure 1. Standard curve of the positive reference theorem. The positive reference serum and five standard dilutions in negative serum were titred to endpoint in PBS ($n = 33$; $r = 0.95$; $p < 0.001$)

Results

Figure 2 demonstrates positive staining of cryostat sections of a sympathetic and a parasympathetic ganglion. In the group of 206 Type 1 DM patients, CF-SG autoantibodies were detected in 47 (23 %). This compares with only 3 (5 %) patients with non-diabetic neurological disease ($p < 0.01$) and 3 (2 %) of 131 healthy control subjects ($p < 0.0001$). Figure 3 shows the individual titres of CF-SG autoantibodies in newly diagnosed and long duration Type 1 DM patients, as well as in patients with non-diabetic neurological disease and control subjects. CF-PSG autoantibodies were observed in 21 (10 %) of 206 Type 1 DM patients compared to 1 (2 %) patient with a non-diabetic neurological disease ($p < 0.05$) and 4 (3 %) healthy control subjects ($p < 0.05$). In the Type 1 DM patients, CF-SG autoantibodies were detected significantly more frequently than CF-PSG autoantibodies ($p < 0.001$). Differences in prevalences of CF-SG or CF-PSG autoantibodies between patients with non-diabetic neurological disease and healthy control subjects were not significant. The diagnoses in patients with non-

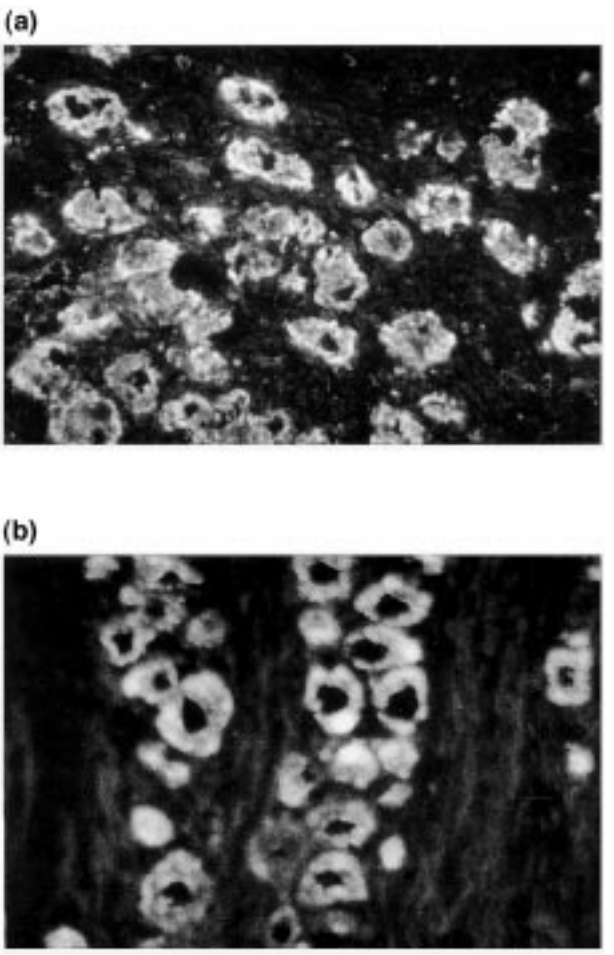

Figure 2. Positive staining of (a) a sympathetic and (b) a parasympathetic ganglion

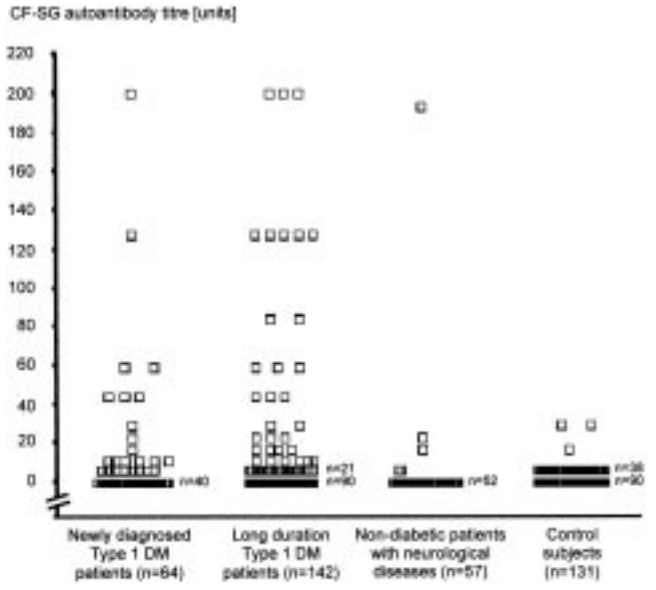

Figure 3. Individual titres of CF-SG autoantibodies in newly diagnosed and long duration Type 1 diabetes mellitus as well as in patients with neurological diseases other than diabetic origin and control subjects

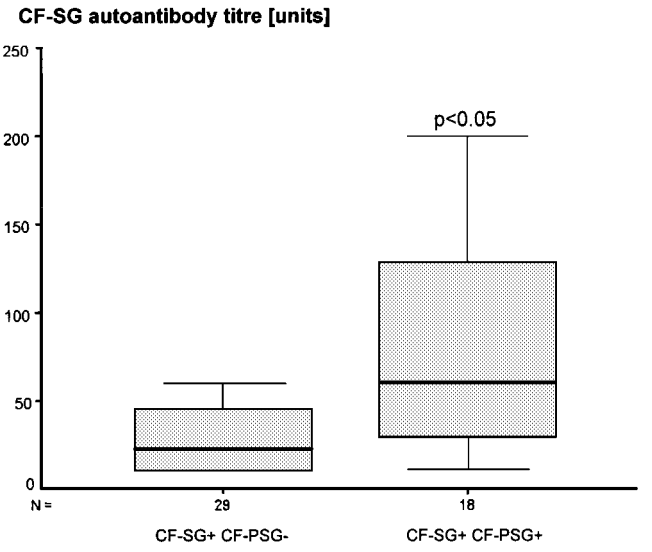

Figure 4. Comparison of CF-SG autoantibody titres between Type 1 DM patients with CF-SG autoantibodies but not CF-PSG autoantibodies (CF-SG+ CF-PSG–) and Type 1 DM patients with both CF-SG and CF-PSG autoantibodies (CF-SG+ CF-PSG+)

diabetic neurological disease, who were positive for either one of the two autoantibodies, is specified in Table 2.

Nineteen (9 %) Type 1 DM patients were positive for both CF-SG and CF-PSG autoantibodies (Table 3). All control subjects with CF-SG autoantibodies ($n = 3$) also had CF-PSG autoantibodies. All Type 1 DM patients with CF-PSG autoantibodies, exept one newly diagnosed and one long duration patient, also demonstrated CF-SG autoantibodies (Table 3). Type 1 DM patients with both CF-SG and CF-PSG autoantibodies had significantly higher CF-SG autoantibody titres (median 60 units) than those, who were positive only for CF-SG autoantibodies (median 23 units, $p < 0.05$; Figure 4). In newly diagnosed and established Type 1 DM patients, frequencies of CF-SG and CF-PSG autoantibodies were not significantly different (Table 3).

Table 3. Prevalences of CF-SG and CF-PSG autoantibodies in the study groups

	CF-SGab	CF-PSGab	CF-SGab and CF-PSGab
Newly diagnosed Type 1 DM patients (*n*(%))	16 (25 %)	7 (11 %)	6 (9 %)
Long-term Type 1 DM patients (*n*(%))	31 (22 %)	14 (10 %)	13 (9 %)
Patients with neurological diseases other than diabetic neuropathy (*n*(%))	3 (5 %)	1 (2 %)	1 (2 %)
Control subjects (*n*(%))	3 (2 %)	4 (3 %)	3 (2 %)

CF-SGab, sympathetic ganglia autoantibodies; CF-PSGab, parasympathetic ganglia autoantibodies.

Of the Type 1 DM patients, 4.7 % newly diagnosed and 40.8 % long duration demonstrated ECG-based CAN. Long duration Type 1 DM patients with ECG-based CAN tended to present more frequently with CF-SG autoantibodies than long-duration Type 1 DM patients without ECG-based CAN, but this did not achieve significance (29 % vs 17 %, $p = 0.06$; Figure 5). Correlating CF-SG autoantibody titres with the individual parameters of cardiac reflex tests in the entire group of Type 1 DM patients, a significant association with the CV of HRV during deep breathing ($r = -0.2$; $P < 0.01$) and the max/min 30:15 ratio during standing from lying ($r = -0.2$; $p < 0.01$), but not with the CV of HRV at rest and the Valsalva ratio was observed. Four (80 %) of 5 newly diagnosed and 23 (32 %) of 73 long duration Type 1 DM patients with either one or both associated tests being abnormal presented with CF-SG autoantibodies compared to 12 (25 %) of 58 newly diagnosed ($p < 0.05$) and 7 (11 %) of 63 long duration Type 1 DM patients

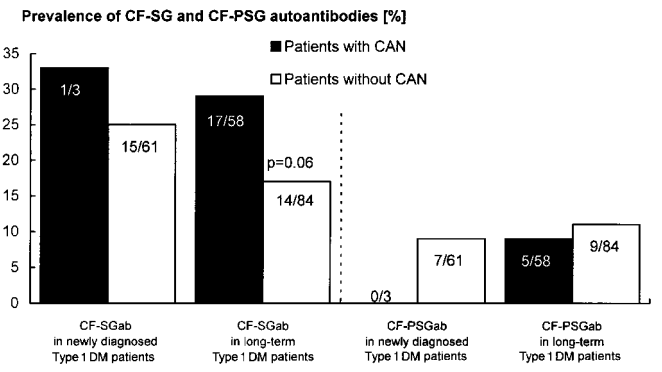

Figure 5. Comparison of prevalences of CF-SG and CF-PSG autoantibodies (ab) between Type 1 DM patients with and without ECG-based cardiac autonomic neuropathy (CAN)

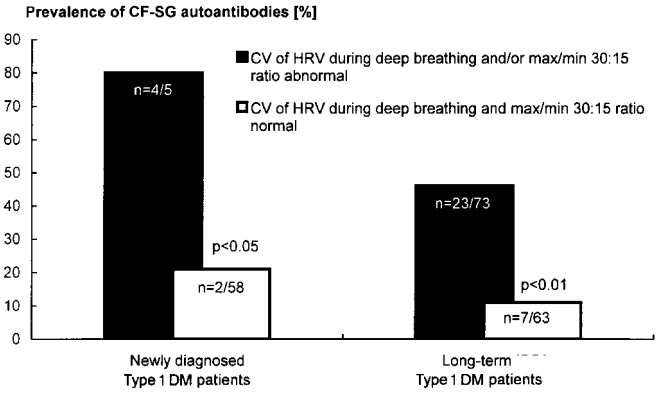

Figure 6. Prevalence of CF-SG autoantibodies in newly diagnosed and long-term Type 1 DM patients with abnormal coefficient of variation (CV) of heart rate variation (HRV) during deep breathing and/or abnormal max/min 30:15 ratio during standing from lying

($p < 0.01$), in whom both tests were normal (Figure 6). In Type 1 DM patients, the presence of CF-PSG autoantibodies was not related to ECG-based CAN (Figure 5) or abnormalities of the individual parameters of cardiac reflex tests. Furthermore, Type 1 DM patients with both CF-SG and CF-PSG autoantibodies did not show abnormalities of either one of the cardiac reflex tests more frequently than patients, who presented with CF-SG but not CF-PSG autoantibodies. CF-SG and CF-PSG autoantibodies were not associated with age, sex or HbA_{1c} in newly diagnosed and long duration Type 1 DM patients nor with duration of diabetes in long duration Type 1 DM patients.

Discussion

The study demonstrates that in patients with Type 1 diabetes mellitus both CF-SG and CF-PSG autoantibodies can be detected more frequently than in healthy control subjects and in patients with non-diabetic neurological disease. In both newly diagnosed and established Type 1 diabetic patients, CF-SG autoantibodies were significantly associated with abnormalities of heart rate variation during deep breathing and during standing from lying.

To our knowledge, CF-PSG autoantibodies have not previously been assessed in Type 1 diabetes mellitus. In this study, their prevalence (10 %) was lower than the prevalence of CF-SG autoantibodies (23 %) and was comparable with investigations of autoantibodies against vagal nerve.[4,5,16] The results do not indicate that CF-PSG autoantibodies are related to cardiac autonomic dysfunction as assessed by cardiac reflex tests. This is consistent with previous reports of a lack of association between autoantibodies against vagal nerve and cardiac reflex tests in Type 1 DM.[5,16] We give further evidence that Type 1 DM patients with diagnosis of ECG-based cardiac autonomic neuropathy (CAN) tend to exhibit CF-SG autoantibodies more frequently than IDDM patients without CAN,[5,8] This did not reach significance in our study but a remarkable observation is the association of CF-SG autoantibodies and abnormal heart rate variation during deep breathing and abnormal heart rate variation from lying to standing. Both cardiac reflex tests have been reported to be reliable indications of diabetic cardiac autonomic dysfunction.[17–19] Applying single-photon emission computed tomography (SPECT) using the tracer 123-I-MIBG to established Type 1 DM patients, we have previously observed a significant association of CF-SG autoantibodies and cardiac sympathetic denervation.[7] This scintigraphic technique is a direct and very sensitive approach for assessment of cardiac sympathetic innervation.[13,20]

Our data suggest that autoantibodies against sympathetic and parasympathetic tissues occur to some extent seperately. One explanation for the formation of autoantibodies is that primary nerve damage occurs due to metabolic and/or microvascular factors and induces an autoimmune response. It is possible that the sympathetic nervous system could be more susceptible to autoimmune phenomena than the parasympathetic nervous system. This argument is supported by the high frequency of scintigraphically assessed cardiac sympathetic abnormalities in newly diagnosed Type 1 DM.[21] The higher titres of CF-SG autoantibodies in Type 1 DM patients with both CF-SG and CF-PSG autoantibodies than in patients with CF-SG but without CF-PSG autoantibodies suggest a progression of the autoimmune response against sympathetic nervous tissue. However, the presence of both CF-SG and CF-PSG autoantibodies together was not associated with a higher frequency of cardiac autonomic dysfunction.

The failure of a significant detection of both CF-SG or CF-PSG autoantibodies in patients with non-diabetic neurological disease suggests that both autoantibodies are specific autoimmune phenomena in Type 1 DM. Interestingly, one non-diabetic neuropathic patient with CF-SG autoantibodies was diagnosed to have thyroiditis, an autoimmune disorder, which has been reported to be associated with Type 1 DM.[22] The specificity of CF-SG and CF-PSG autoantibodies to Type 1 DM is also underlined by the observation that Type 2 DM patients exhibit neither CF-SG nor CF-PSG autoantibodies to a significant extent.[23,24]

The present study gives further evidence for CF-SG autoantibodies being involved in cardiac autonomic dysfunction in Type 1 DM. The additional assessment of CF-PSG autoantibodies does not increase the predictive value with regard to the presence of ECG-based cardiac autonomic neuropathy. Prospective studies are required to investigate the role of CF-SG autoantibodies to predict the later occurrence of cardiac autonomic abnormalities in Type 1 DM.

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